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Two novel homologous proteins of *Streptomyces coelicolor* and *Streptomyces lividans* are involved in the formation of the rodlet layer and mediate attachment to a hydrophobic surface

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Summary

The filamentous bacteria *Streptomyces coelicolor* and *Streptomyces lividans* exhibit a complex life cycle. After a branched submerged mycelium has been established, aerial hyphae are formed that may septate to form chains of spores. The aerial structures possess several surface layers of unknown nature that make them hydrophobic, one of which is the rodlet layer. We have identified two homologous proteins, RdlA and RdlB, that are involved in the formation of the rodlet layer in both streptomycetes. The *rdl* genes are expressed in growing aerial hyphae but not in spores. Immunolocalization showed that RdlA and RdlB are present at surfaces of aerial structures, where they form a highly insoluble layer. Disruption of both *rdlA* and *rdlB* in *S. coelicolor* and *S. lividans* (Δ*rdlAB* strains) did not affect the formation and differentiation of aerial hyphae. However, the characteristic rodlet layer was absent. Genes *rdlA* and *rdlB* were also expressed in submerged hyphae that were in contact with a hydrophobic solid. Attachment to this substratum was greatly reduced in the Δ*rdlAB* strains. Sequences homologous to *rdlA* and *rdlB* occur in a number of streptomycetes representing the phylogenetic diversity of this group of bacteria, indicating a general role for these proteins in rodlet formation and attachment.

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Introduction

Streptomycetes are Gram-positive soil bacteria that colonize moist substrates by forming a branched network of multinucleoid hyphae. At some stage during their life cycle, these bacteria are confronted with a hydrophobic environment. For instance, after a feeding substrate mycelium has been established, hyphae leave the aqueous environment to grow into the hydrophobic air. These aerial hyphae differentiate further by forming chains of uninucleoid cells, which metamorphose into pigmented spores. Spores or hyphae of streptomycetes may also encounter hydrophobic solids such as surfaces of dead or living organisms. When streptomycete hyphae leave their aqueous environment, they change their surface. Hyphae in a moist substrate are hydrophilic, whereas the surfaces of aerial hyphae and spores are hydrophobic.

Formation of aerial structures has been best studied in *Streptomyces coelicolor* (for recent reviews, see Chater, 1998; 2001; Kelemen and Buttner, 1998; Wösten and Willey, 2000). Bald (*bld*) mutants of *S. coelicolor* were isolated that, when grown on rich medium, are affected in the formation of aerial structures and in the production of a small surface-active peptide called SapB (Willey *et al*., 1991). Many of these mutants appear to be affected in an extracellular signalling cascade involved in the erection of aerial hyphae (Willey *et al*., 1993; Nodwell *et al*., 1996; 1999). Experimental evidence suggests the existence of at least five signalling molecules. It was hypothesized that each signal triggers the synthesis and release of the next signal, ultimately leading to the production and secretion of SapB (Willey *et al*., 1993; Nodwell *et al*., 1996). By lowering the water surface tension from 72 to 32 mJ m^{-2}, SapB enables hyphae to breach the water–air interface to grow into the air (Tillotson *et al*., 1998).

Aerial hyphae and spores of *S. coelicolor* have several surface layers that make them hydrophobic. One surface layer, called the rodlet layer, has a typical ultrastructure of a mosaic of 8- to 10-nm-wide parallel rods (Wildermuth *et al*., 1971; Smucker and Pfister, 1978). The nature of the surface layers is not known. SapB is not expected to form one of these layers, as this peptide was localized in the culture medium but could not be detected at the surfaces of aerial structures (Wösten and Willey, 2000).

The life cycle of filamentous fungi is very similar to that of the streptomycetes. They also form hydrophobic reproductive structures (e.g. aerial hyphae, fruiting bodies such as mushrooms, and spores) with rodlet-decorated surfaces. In this case, a film of highly insoluble self-assembled class I hydrophobin is responsible for this typical ultrastructure and the surface hydrophobicity (Wösten *et al*., 1993; 1994). These proteins also mediate attachment to hydrophobic solids (Wösten *et al*., 1993; 1994), such as to the surface of a host of a pathogenic fungus (Talbot *et al*., 1993; 1996). The latter is important during the initial stages of infection.

We adopted here the protocol used previously to extract class I hydrophobins selectively from fungal aerial structures (Wessels *et al*., 1991a,b; de Vries *et al*., 1993). This protocol is based on the insolubility of self-assembled class I hydrophobins in hot 2% SDS and their solubility in trifluoroacetic acid (TFA). Using this method, we identified two homologous proteins, designated rodlins (Rdl), that are involved in the formation of the rodlet layer on aerial structures of *S. lividans* and *S. coelicolor* and that also mediate attachment to hydrophobic surfaces.

Results

Identification of an abundant SDS-insoluble cell wall protein specifically present in aerial structures of S. coelicolor *and* S. lividans

Cell walls from 5-day-old sporulating cultures of *S. coelicolor* and *S. lividans* grown on solid medium were treated with 2% SDS at 100°C. The SDS-extracted cell walls were washed with water, lyophilized and extracted with TFA. SDS–PAGE of the SDS-soluble fraction showed a complex pattern of polypeptides (results not shown). Among the cell wall proteins of *S. coelicolor* and *S. lividans* that were insoluble in hot SDS but soluble in TFA, an abundant polypeptide, called Rdl, was observed with an apparent molecular weight of 18 kDa (Fig. 1A, lane 2). This protein was absent in a TFA extract of SDS-treated cell walls from a 3-day-old liquid shaken culture of *S. lividans* (Fig. 1A, lane 1). Western analysis with antibodies raised against Rdl showed the absence of Rdl in the SDS-soluble fraction of cultures of both streptomycetes grown in liquid or on solid medium (results not shown).

The presence of Rdl correlated with the presence of aerial hyphae. Rdl was absent in cell walls from 1-day-old surface-grown cultures that had not yet formed aerial hyphae (Fig. 1B). Similarly, Rdl was absent in cell walls from 1- to 7-day-old cultures of the *bld* mutants *bld261*, *bldD* and *bldH* of *S. coelicolor* grown on solid or liquid medium (results not shown). In contrast, the protein was abundantly present in cultures of *S. lividans* TK23 that had formed a confluent layer of aerial hyphae on solid medium after 2 days of growth. The amount of Rdl did not change during the following 5 days when aerial hyphae differentiated further to form chains of spores (Fig. 1B). Similar results were obtained with *S. coelicolor* M145 and *S. coelicolor whiG*, although aerial hyphae formation was delayed compared with *S. lividans* (see below).

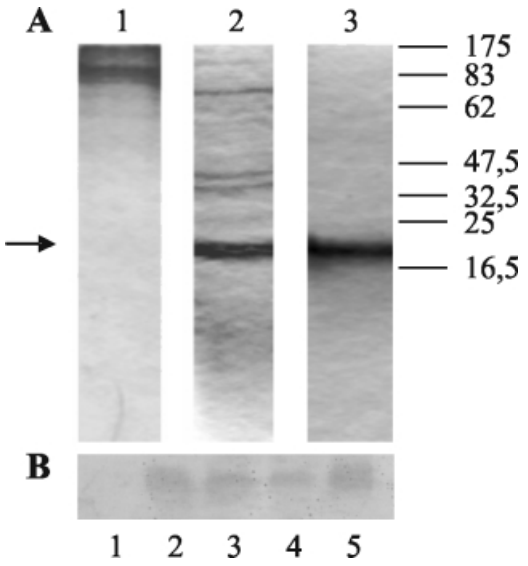

Fig. 1. Rdl is a protein present in cultures of *S. coelicolor* and *S. lividans* forming aerial structures.
A. SDS–PAGE analysis of TFA extracts of SDS-treated cell walls from a 3-day-old liquid shaken culture (lane 1) and a 5-day-old sporulating culture of *S. lividans* (lane 2). Rdl extracted with TFA from the latter culture is the main protein that is soluble in water (lane 3). Arrow indicates the position of Rdl. Molecular weights are given in kDa.
B. Western analysis of TFA extracts of SDS-treated walls from 1-day-old (lane 1), 2-day-old (lane 2), 3-day-old (lane 3), 4-day-old (lane 4) and 7-day-old (lane 5) cultures of *S. lividans* grown on solid medium using antibodies raised against Rdl. Cultures formed aerial hyphae at day 2 and started to sporulate at day 3. Similar results were obtained with *S. coelicolor*.

When water was added instead of 2% SDS to a TFA extract of SDS-treated cell walls from a culture forming aerial hyphae, Rdl was the main protein that dissolved (Fig. 1A, lane 3). However, the protein formed an SDS-insoluble complex upon shaking. The complex could be dissociated with TFA. These data indicate that, under physiological conditions, Rdl is an SDS-insoluble cell wall protein present in cultures of *S. coelicolor* and *S. lividans* forming aerial structures.

Cloning and characterization of the rdl *genes of* S. coelicolor *and* S. lividans

N-terminal sequencing revealed that the Rdl protein band running at the 18 kDa position was in fact a mixture of two similar proteins, called RdlA and RdlB, with slightly different N-termini. In addition, the N-termini of two internal peptides were determined that resulted from a

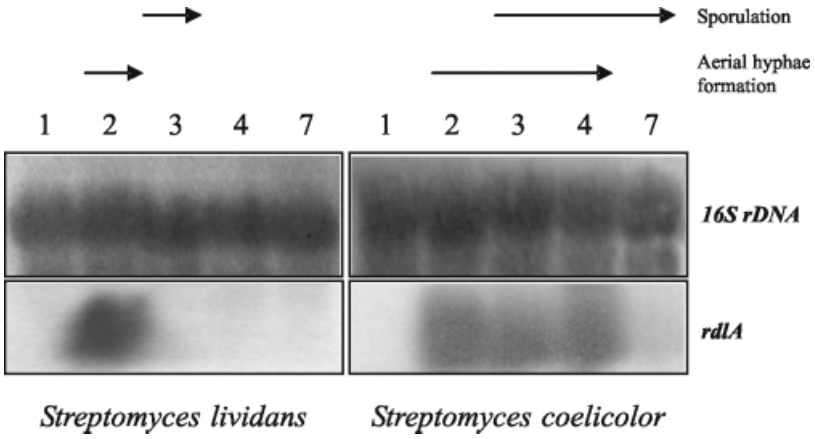

Fig. 2. Temporal expression of *rdlA* in *S. lividans* and *S. coelicolor* grown on solid medium. Northern blots were rehybridized with 16S rDNA to confirm that lanes contained equal amounts of RNA. Identical results were obtained using the coding sequence of *rdlB* as a probe.

tryptic digestion of a mixture of RdlA and RdlB. A radioactive degenerated oligonucleotide based on one of the peptides was used to screen the cosmid library of *S. coelicolor* (Redenbach *et al*., 1996). The oligonucleotide hybridized to the overlapping cosmids C46 and C61. The hybridizing fragment of C46 was contained on a 4.5 kb *Sal*I fragment. This fragment was cloned in the pBluescript KS+ *Sal*I site and partially sequenced. An open reading frame (ORF) was identified that encodes a putative polypeptide of 131 amino acids, starting with a putative signal sequence for secretion of 28 amino acids followed by a sequence corresponding to the determined N-terminal sequence of RdlA as found in the cell wall (mature RdlA). The N-terminal sequences of both internal peptides were also identified in the ORF. The *rdlB* gene, divergently transcribed from *rdlA*, was identified 262 bp upstream of the start codon of *rdlA*. It encodes a protein very similar to that encoded by *rdlA* (68.7% identity, 83.2% similarity; see accession numbers AJ315950 and AJ315951) and contains the determined N-terminus of mature RdlB preceded by a putative signal sequence of 28 amino acids.

The coding sequences of *rdlA* and *rdlB* hybridized to the same unique fragments of genomic DNA of *S. coelicolor* and *S. lividans* digested with a variety of enzymes. For instance, a 4.5 kb *Sal*I fragment of genomic DNA from *S. coelicolor* and *S. lividans* hybridized to both *rdlA* and *rdlB*. A slightly larger genomic fragment hybridized after digestion with *Blp*I, whereas digestion with *Pst*I resulted in a fragment of about 8 kb (data not shown). The complete genome sequence of *S. coelicolor* (http://www.sanger.ac.uk/Projects/S_coelicolor/) did not reveal other sequences homologous to *rdlA* and *rdlB*.

Using polymerase chain reaction (PCR) and primers based on *rdlA* and *rdlB* of *S. coelicolor*, the homologues of *S. lividans* were isolated. Their sequences were identical to those of *S. coelicolor*. Genomic DNA from *Streptomyces tendae*, *Streptomyces griseus* and the potato pathogen *Streptomyces scabies* hybridized with probes directed against the coding sequences of *rdlA* and *rdlB* (data not shown). Genomic DNA from actinomycetes not belonging to the streptomycetes, i.e. *Amycolatopsis mediterranei* and *Rhodococcus erythropolis*, did not hybridize with either probe, even under low stringency (data not shown).

rdlA *and* rdlB *are expressed in aerial hyphae*

Total RNA was isolated from cultures of *S. coelicolor* M145 and *S. lividans* TK23 grown in liquid or solid medium. After separation on a formaldehyde gel and blotting to a nylon membrane, RNA was hybridized with a probe representing the coding sequence of *rdlA* (Fig. 2) or *rdlB* (not shown).

Accumulation of mRNA from the *rdl* genes in *S. lividans* was only observed at day 2, coinciding with the formation of a confluent layer of aerial hyphae (Fig. 2). No accumulation of *rdl* mRNAs was observed in 1-day-old cultures growing submerged only or in 3-day-old sporulating cultures. mRNA from the *rdl* genes in *S. coelicolor* accumulated at days 2–4. Formation of aerial hyphae in this streptomycete was delayed compared with that in *S. lividans*. It started at day 2, but a confluent layer was not observed until day 4. As a consequence, the formation of aerial hyphae and sporulation partially overlapped (Fig. 2). From these data and the fact that accumulation of *rdl* mRNA was not observed in liquid shaken cultures throughout growth (not shown), we conclude that *rdl* expression correlated with the formation of aerial hyphae.

To determine the spatial expression of *rdlA* and *rdlB*, both orientations of the 262 bp intergenic region of the *rdl* coding sequences were cloned in vector pIJ8630 in front of the coding sequence of an enhanced green fluorescent protein (eGFP) with an adapted codon usage for *S. coelicolor* and *S. lividans* (Sun *et al*., 1999). This resulted in plasmids pIJ8630*a* and pIJ8630*b*. Spores from wild-type strains of *S. coelicolor* and *S. lividans* and

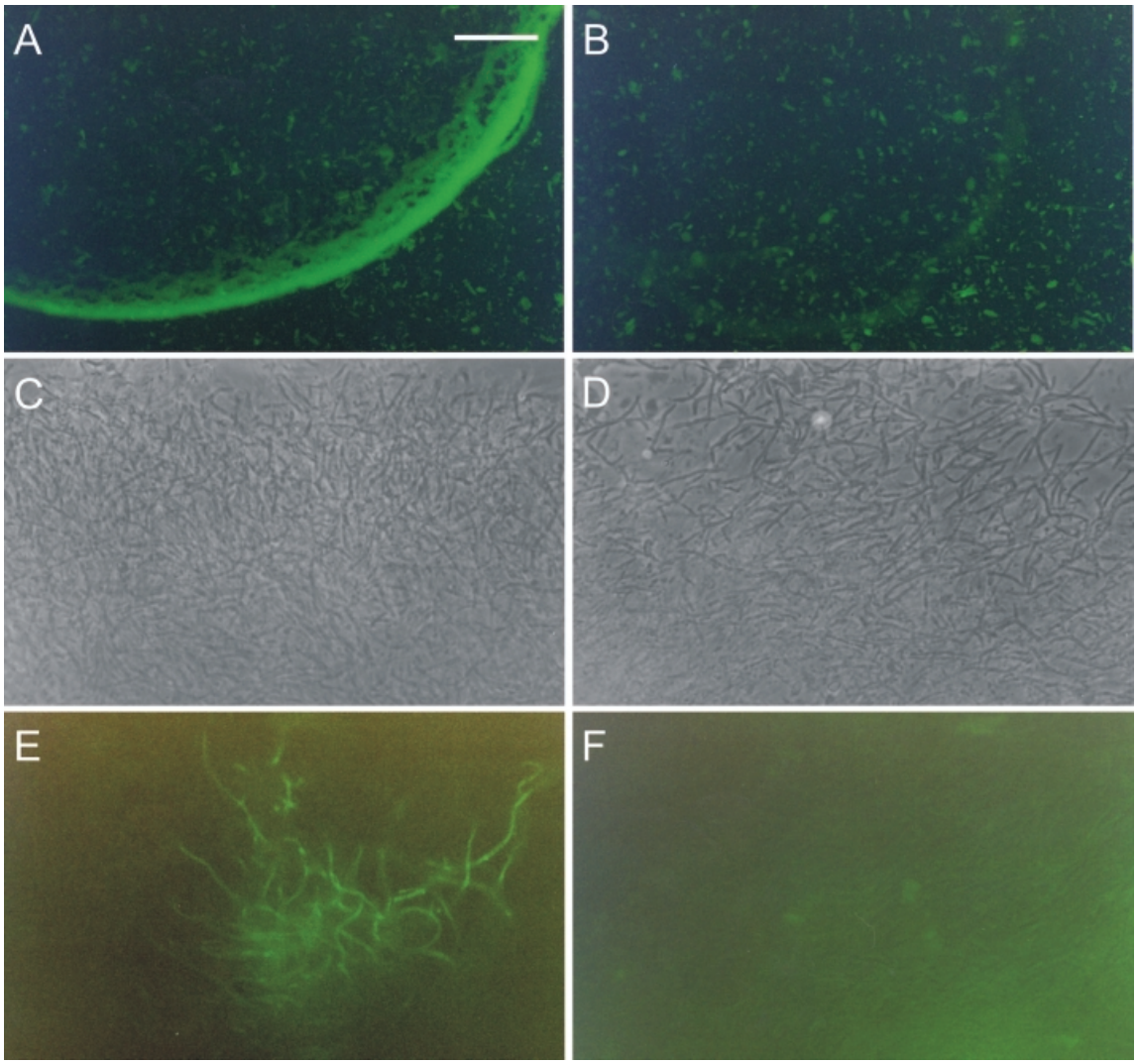

Fig. 3. Spatial expression of *rdlA* in 2-day-old-cultures of *S. lividans* grown on solid medium visualized using eGFP as a reporter. Fluorescence of GFP was observed in transformants in a zone where aerial hyphae were formed (A), but not in a wild-type colony (B). At higher magnification, light microscopy showed a dense mycelium in the zone where the transformant (C) and the wild type (D) formed aerial hyphae. However, fluorescence of GFP was restricted to aerial hyphae of transformants being focused in the upper plane of the sample (E), wild-type aerial hyphae serving as a negative control (F). Identical results were obtained with an *S. lividans* strain expressing eGFP under the control of the *rdlB* promoter. Bar represents 500 μm (A and B) and 25 μm (C and D).

transformants containing either construct were inoculated as a lawn on an object glass with a thin layer of agar medium. It appeared that wild-type strains of *S. coelicolor* were highly autofluorescent, but autofluorescence of *S. lividans* was negligible. Fluorescence in colonies of *S. lividans* transformed with either eGFP construct was observed at the outer part of the colony after 2 days of growth, correlating with the area in which aerial hyphae were formed (Fig. 3A), and was absent in wild type (Fig. 3B). When growth was prolonged, fluorescence in this zone decreased to wild-type levels but increased in the central zone, coinciding with the formation of aerial hyphae. At higher magnification, it was observed that aerial hyphae but not submerged hyphae were fluorescent (Fig. 3E). No fluorescence was observed in the wild-type strain at this magnification (Fig. 3F). These results show that the *rdl* genes are expressed in developing aerial hyphae.

RdlA and RdlB are localized at the outer surface of aerial hyphae and spores

RdlA and RdlB were localized using an antiserum raised against a mixture of RdlA and RdlB from *S. lividans*. Immunolabelling was observed at the outer surface of aerial hyphae and spores of *S. lividans* and *S. coelicolor* (Fig. 4B). Some label was also found within the cell walls of the aerial structures. The reactive layer at the outer

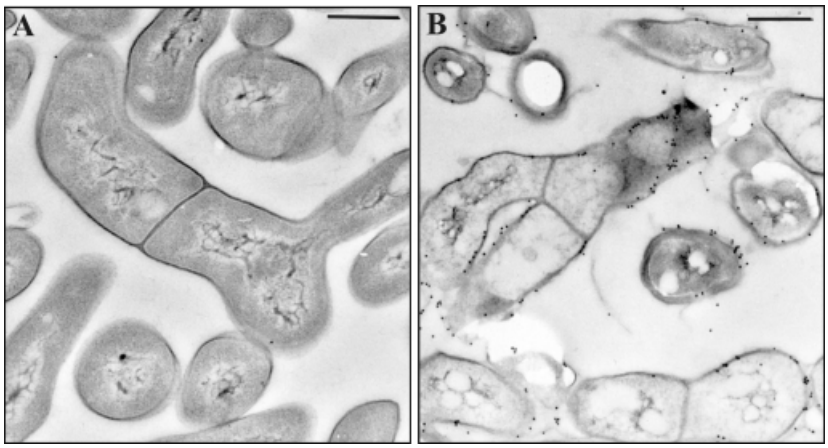

Fig. 4. Immunolocalization of RdlA and RdlB in submerged (A) and aerial (B) hyphae of *S. coelicolor*. Bars represent 0.5 μm.

surface was sometimes detached, indicating that it is a discrete layer. The antiserum reacted neither with submerged hyphae of wild-type strains of *S. coelicolor* and *S. lividans* (Fig. 4A) nor with hyphae of the *bld261*, *bldD* and *bldH* mutants of *S. coelicolor*. In contrast, aerial hyphae of a *whiG* mutant of *S. coelicolor* were labelled (data not shown).

Disruption of rdlA *and* rdlB *does not affect the formation of aerial structures but does affect the formation of the rodlet layer*

As expression profiles of *rdlA* and *rdlB* were similar, these genes may be redundant. Therefore, both genes were inactivated in *S. coelicolor* M145 and *S. lividans* TK23 using deletion construct pC46d. The complete coding sequence of *rdlA*, most of the coding sequence of *rdlB* and the intergenic region were replaced by a hygromycin B resistance cassette. Gene replacement was confirmed by Southern analysis. To exclude interference from the replacement of *rdlA* and *rdlB* with transcription of upstream and downstream genes, Northerns were probed with ORF SCC46.02c located 288 bp upstream of *rdlA* and *rdlB* and ORF SCC46.05c located 89 bp downstream of these genes. Accumulation of mRNA was similar in wild-type and disruptant strains grown on solid media.

Germination of spores, growth rates and differentiation of aerial hyphae into spores were similar in wild-type and Δ*rdlAB* strains using a variety of media and culture conditions (data not shown). In addition, no difference could be observed in the viability of spores after freeze-drying or drying spores in the air. Surface hydrophobicity was also unaffected (van der Mei *et al*., 1991). Wild-type strains of *S. coelicolor* and *S. lividans* showed water contact angles of 124 ± 5°, whereas those of disruptant strains were 133 ± 6° and 126 ± 3° respectively.

To analyse whether the disruption of *rdlA* and *rdlB* affects the formation of the rodlet layer at surfaces of aerial hyphae and spores, wild-type and Δ*rdlAB* strains were analysed using scanning electron microscopy. In contrast to the wild-type strains, no rodlets were observed at the surfaces of aerial hyphae and spores from *S. coelicolor* Δ*rdlAB6* (Fig. 5) and *S. lividans* Δ*rdlAB3* (data not shown). Integration of the 4.5 kb *Sal*I fragment encompassing both *rdl* genes into the genomic *attP* site of the null mutants of *S. coelicolor* and *S. lividans* restored rodlet formation (see Fig. 5).

Disruption of the rdl *genes affects the attachment of hyphae to polystyrene*

Expression of *rdlA* and *rdlB* in hyphae confronted with a hydrophobic solid was studied by growing *S. lividans* strains transformed with plasmid pIJ8630*a* or pIJ8630*b* (see above) in 96-well plates in liquid medium without shaking, followed by analysis of GFP expression. Under this condition, no autofluorescence was observed. Hyphae not in contact with the hydrophobic surface of the microtitre plate were not fluorescent throughout culturing (Fig. 6C). In contrast, hyphae in contact with the solid did express eGFP (Fig. 6D).

A role for RdlA and RdlB in attachment was studied by growing cultures in microtitre plates, followed by staining with crystal violet and thorough washing to remove all unattached cells. Throughout culturing, attachment of *S. coelicolor* Δ*rdlAB6* was only 10–50% compared with that of the wild-type strain (Fig. 6A and B). Similar results were obtained with *S. lividans* (data not shown). Attachment of the Δ*rdlAB* strains could not be restored by integrating the 4.5 kb *Sal*I fragment encompassing both *rdl* genes into the genomic *attP* site. As the reason for this was not clear, two additional independent null mutants were analysed for their capacity to adhere to the microtitre plate. Similar results were obtained to those with Δ*rdlAB6* and Δ*rdlAB3* confirming that the rodlins are involved in attachment.

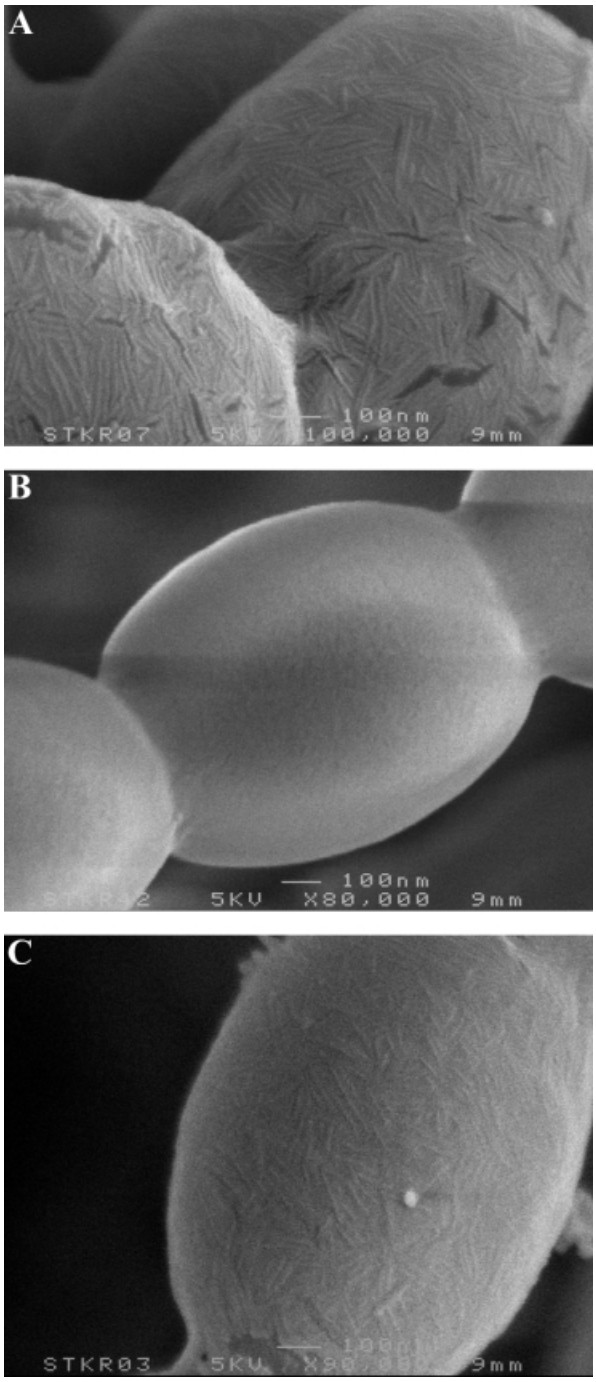

Fig. 5. Scanning electron microscopy revealed that rodlets are present on spores of a wild-type strain of *S. coelicolor* (A) but are absent in *S. coelicolor* Δ*rdlAB6* (B). Rodlet formation was restored in a genetically complemented strain of *S. coelicolor* Δ*rdlAB6* (C). Similar results were obtained with *S. lividans* Δ*rdlAB3*. Bars represent 100 nm.

Discussion

The lifecycle, the mode of growth and the ecological niches of streptomycetes are remarkably similar to those of filamentous fungi. Yet, these microbes belong to different kingdoms that diverged early in evolution. Spores of both groups germinate and form a mycelium that colonizes moist substrates. This mycelium consists of filaments that are surrounded by rigid walls and grow at their apices. After a submerged feeding mycelium has been established, filaments may leave the substrate to form spore-bearing aerial structures. The aerial structures of most species are hydrophobic and characterized by rodlet-decorated surfaces.

The formation of aerial hyphae has been described as a two-step process (Wösten *et al*., 1999). Although oversimplified, given the genetic complexity of this differentiation process, this model is a means to begin to understand aerial growth. In the first step, the water surface tension is dramatically reduced from 72 to 32 mJ m^{-2}, enabling hyphae to breach the colony surface–air interface (Wösten *et al*., 1999). In the second step, the aerial hyphae are coated with a hydrophobic rodlet layer. In filamentous fungi it has been shown that hydrophobins both lower the surface tension and form the rodlet-decorated hydrophobic coating (Wösten *et al*., 1993; 1994a; 1999; 2001). Filamentous bacteria appear to have evolved different molecules to lower the surface tension and to coat the aerial hyphae (Wösten and Willey, 2000). *S. coelicolor* lowers the water surface tension by secreting a small surface-active peptide called SapB (Tillotson *et al*., 1998; Willey *et al*., 1991; 1993). We identified here the proteins that form the rodlet layer. So far, this is the first example of structural proteins coating aerial structures of filamentous bacteria. These proteins, called rodlins, were isolated adopting the procedure used selectively to extract the hydrophobins from cell walls of fungal aerial structures. Despite their remarkable resemblance in solubility characteristics, rodlins are not related to the fungal hydrophobins. Apparently, distinct proteins can form a surface layer with a similar ultrastructural appearance. The rodlet layers found in streptomycetes are probably all formed by rodlins, as the encoding genes in *S. lividans* and *S. coelicolor*, *rdlA* and *rdlB*, hybridized to genomic DNA from five different streptomycetes representing the phylogenetic diversity of this group of bacteria.

Inactivation of the *SC3* hydrophobin gene in *S. commune* affected the formation of aerial hyphae. Those aerial hyphae formed were hydrophilic (van Wetter *et al*., 1996). In contrast, deletion of both *rdl* genes in *S. coelicolor* and *S. lividans* neither affected the formation of aerial hyphae nor surface hydrophobicity. Apparently, the rodlet layer is not involved in the formation of aerial hyphae. This can be explained by the fact that SapB mediates escape of hyphae into the air by lowering the water surface tension whereas, apart from the rodlin layer, other layers render aerial hyphae hydrophobic. Hydrophobins have been shown to mediate the attachment of fungal hyphae to hydrophobic surfaces (Wösten *et al*., 1994b),

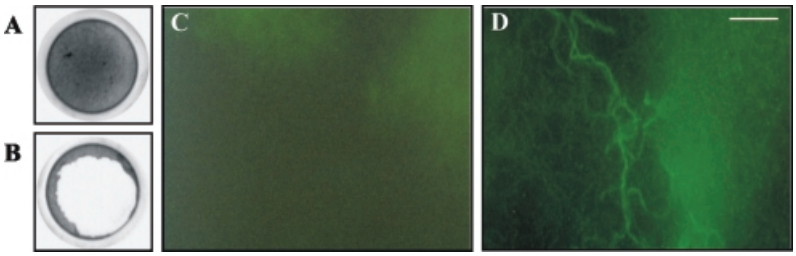

Fig. 6. *S. coelicolor* wild-type strain M145 attaches more strongly to a polystyrene microtitre plate (A) than strain Δ*rdlAB6* (B), as visualized by staining attached hyphae with crystal violet. Similar results were obtained with *S. lividans*. Expression of *rdlA* and *rdlB* in *S. lividans* during attachment was studied using eGFP as a reporter. Fluorescence was not observed in hyphae that were loosely, if at all, attached (C), whereas GFP accumulated in hyphae firmly attached to the polystyrene surface (D). Bar represents 25 μm.

such as the hydrophobic surface of a host of a plant pathogen (Talbot *et al*., 1993; 1996). Attachment to a hydrophobic surface was also strongly decreased in Δ*rdlAB* strains of *S. coelicolor* and *S. lividans.* Yet, by expressing the *rdl* genes at the *attP* locus in the genome, attachment could not be complemented. The reason for this is not yet clear but may result from different expression levels at this ectopic site, interfering with the proper formation of the attaching layer. Adhesion of streptomycetes to hydrophobic surfaces may play a role during invasive growth of wood being rich in hydrophobic lignin. In pathogenic streptomycetes (e.g. the potato pathogen *Streptomyces scabies*), homologues of RdlA and RdlB may be instrumental in pathogenicity by attaching the pathogen to the host.

Experimental procedures

Strains and plasmids

Escherichia coli strains DH5α or JM110 were used for cloning purposes. *S. coelicolor* strains M145 (Kieser *et al*., 2000), J1700 (*hisA1*, *uraA1*, *strA1*, *bldA39*, *Pgl*$^-$) (Piret and Chater, 1985), J774 (*mthB2*, *cysD18*, *pheA1*, *agaA7*, *strA1*, *bldD*, NF, SCP2) (Merrick, 1976), C109 (*hisA1*, *uraA1*, *strA1*, *bldH109*, *Pgl*$^-$) (Champness, 1988) and J1820 (*hisA1*, *uraA1*, *strA1*, *whiG71*, *Pgl*$^-$) (Méndez and Chater, 1987) were used as well as *S. lividans* TK23 (Kieser *et al*., 2000), *Streptomyces tendae* Tü901/8c (Richter *et al*., 1998) and *Streptomyces griseus* (DSMZ 40236). Chromosomal DNA from *Streptomyces scabies* was kindly provided by Professor E. M. H. Wellington (University of Warwick, UK). Vectors and constructs are summarized in Table 1.

Growth conditions and media

Streptomyces strains were grown at 30°C on solid MS agar medium or in YEME medium as liquid shaken cultures (Kieser *et al*., 2000). The solid medium R2YE (Kieser *et al*., 2000) was used for regenerating protoplasts. To assess attachment to a hydrophobic solid, *S. coelicolor* and *S. lividans* were grown in NMMP (Kieser *et al*., 2000) in the absence of PEG 6000 and using 50 mM glucose as a carbon source in 96-well flat-bottomed microtitre plates (Costar, Corning). Before inoculation, spores, stored at –20°C in 20% glycerol, were taken up in NMMP to a final concentration of 5×10^6 spores ml^{-1}. Flat-bottomed 96-well microtitre plates were filled with 200 μl of spore suspension per well.

Molecular techniques

Standard molecular techniques followed the methods described by Sambrook *et al*. (1989). Protoplast preparation

Table 1. Vectors and constructs.

Plasmid	Description	Reference
pIJ8600	*E. coli–Streptomyces* shuttle vector with pUC18 *ori*, *oriT* and *attP* site. It integrates at the φC31 attachment site in *S. coelicolor* and *S. lividans*	Sun *et al*. (1999)
pIJ8630	*E. coli–Streptomyces* shuttle vector with pUC18 *ori*, *oriT* and *attP* site containing an *eGFP* gene adapted for codon usage in streptomycetes. It integrates at the φC31 attachment site in *S. coelicolor* and *S. lividans*	Sun *et al*. (1999)
pIJ8630*a*	pIJ8630 containing the 262 bp *S. coelicolor* promoter region of *rdlA* with an *Nde*I site at the 3′ end allowing translational fusions	This work
pIJ8630*b*	As pIJ8630*a* but with the promoter region of *rdlB*	This work
pC46a	pBluescript KS+ (Stratagene) containing a 4.5 kb *Sal*I fragment of cosmid C46 (Redenbach *et al*., 1996) of *S. coelicolor* encompassing the coding sequences of *rdlA* and *rdlB*, their interspersed promoter region and flanking regions of 0.8 kb (*rdlA*) and 2.5 kb (*rdlB*)	This work
pC46b	pZErO-2.1 (Invitrogen) containing the *Sal*I fragment described for pC46a	This work
pC46c	pC46b derivative carrying a 1.4 kb *Sma*I fragment containing a hygromycin resistance cassette (Zalacaín *et al*., 1986) replacing a 0.8 kb *Blp*I–*Sca*I fragment encompassing *rdlA*, the 5′ end of the coding sequence of *rdlB* as well as their interspersed promoter region	This work
pC46d	pC46c derivative with a 1.8 kb *Sma*I fragment containing an apramycin resistance cassette (Prentki and Krisch, 1984) cloned in the *Xba*I site of pC46c	This work
pSET-C46a	pSET152 derivative (Bierman *et al*., 1992) containing the 4.5 kb *Sal*I fragment described for pC46a	This work

and transformation were performed as described by Kieser *et al*. (2000) using alkali-denatured DNA (Oh and Chater, 1997). Chromosomal DNA of *S. coelicolor* and *S. lividans* was isolated according to the method of Verhasselt *et al*. (1989) and modified by the method of Nagy *et al*. (1995). Total RNA of *S. coelicolor* and *S. lividans* was isolated using the SV Total RNA isolation system (Promega) according to the method of Veenendaal and Wösten (1998). DNA and RNA were blotted on nylon filters (Boehringer Mannheim) and hybridized under conditions described by Church and Gilbert (1984) at 60°C. Under these conditions, *rdlA* and *rdlB* do not cross-hybridize. Radioactively labelled probes were made using the oligolabelling kit (Pharmacia).

Isolation of the rdlA *and* rdlB *genes from* S. coelicolor *and* S. lividans

To isolate *rdl* genes from *S. coelicolor* M145 and *S. lividans* TK23, a degenerate oligonucleotide (SGCSGASAGSACS GASAGGTCCTCSAGSACGTGSGASAGSGCGCCGTC) representing the N-terminal sequence of the carboxy-terminal internal peptide of RdlA of *S. lividans* (see *Results*) was radioactively labelled and hybridized to the cosmid library of *S. coelicolor* (Redenbach *et al*., 1996). Accession numbers for *rdlA* and *rdlB* are AJ315950 and AJ315951 respectively.

Construction of the ΔrdlAB *gene deletion plasmid pC46d*

The *rdl* genes were deleted by replacing a 0.8 kb *Blp*I–*Sca*I fragment of pC46b (see Table 1), containing the entire coding sequence of *rdlA* and 136 bp of that of *rdlB* as well as the interspersed promoter region, with a 1.4 kb *Sma*I fragment encompassing the hygromycin B resistance cassette (Zalacaín *et al*., 1986). This resulted in vector pC46c. To select for double cross-over events, plasmid pC46d was made by introducing a 1.8 kb *Sma*I fragment containing an apramycin resistance cassette (Prentki and Krisch, 1984) in the *Xba*I site of pC46c.

Preparation of cell walls and protein extracts

Filaments of *S. coelicolor* and *S. lividans* were fragmented at 20 000 p.s.i. using an SLM French pressure cell press. The homogenate was treated with 2% SDS for 10 min at 100°C, after which the cell walls were fractionated from the cytoplasmic content by centrifugation at 10 000 *g* for 10 min. The cell wall fraction was extracted with hot 2% SDS once more, washed extensively with water and freeze dried. SDS-treated cell walls were then extracted with TFA (Wösten *et al*., 1993). After evaporating the solvent by a stream of air, extracts were taken up in SDS sample buffer (2% SDS, 20% glycerol, 0.02% bromophenol blue, 0.1 M Tris-HCl, pH 6.8, and 5% β-mercaptoethanol) and subjected to SDS–PAGE. If necessary, adjustments in pH were done by the addition of 25% ammonia. RdlA and RdlB were purified by taking up TFA extracts of SDS-treated cell walls in water without shaking. Insolubles were removed by centrifugation at 10 000 *g* for 15 min.

Gel electrophoresis and Western blotting

SDS–PAGE was performed in 16% gels according to the method of Laemmli (1970). Prestained broad-range molecular weight markers from Bio-Rad were used. After separation, proteins were stained with 0.25% Coomassie brilliant blue G-250 (CBB) or blotted onto a polyvinylidene difluoride (PVDF) membrane using semi-dry blotting. For N-terminal sequencing, a PVDF membrane was stained with CBB, and a slice of the membrane containing the protein was excised. After destaining with 30% methanol, the N-terminal sequence was determined using a pulse liquid sequencer on line connected to a PTH analyser (Eurosequence). To determine N-terminal sequences of internal peptides, the protein was eluted from the SDS–PAA gel followed by tryptic digestion. Peptides were sequenced after separation on a C18 reversed phase high-performance liquid chromatography (HPLC) column.

Polyclonal antibodies were raised against a mixture of the *S. lividans* RdlA and RdlB proteins, eluted from an SDS–PAA gel. Antibodies were purified with an acetone powder of mycelium from a liquid shaken culture (Harlow and Lane, 1988). PVDF membranes were treated with diluted anti-RdlA/RdlB serum (1:1000) as described previously (Harlow and Lane, 1988).

Immunolocalization

Fixation, embedding and immunolabelling of cultures were performed as described previously (Wösten *et al*., 1994) with the modification that K4M was substituted for Unicryl. Sectioned material was examined in a Philips CM10 electron microscope. Photographs were made on FGP Kodak film.

Other electron microscopic techniques

For cryo scanning electron microscopy (SEM), sporulating cultures grown on solid MS medium were frozen in a mixture of liquid and solid nitrogen and sputter coated with gold–palladium. Examination was done at 5.0 kV in a Jeol field emission scanning electron microscope type 6301F.

Attachment assay

To quantify attachment of *S. coelicolor* and *S. lividans* to the surface of polystyrene microtitre plates, 25 μl of 0.5% crystal violet (Acros Organics) was added to each well and incubated for 10 min to stain cell material. Wells were washed vigorously with water using a Vaccu-Pette/96 (Sigma), removing all non-adherent cells. After drying overnight at 30°C, the crystal violet associated with the attached material was dissolved in 200 μl of 10% SDS (Reynolds and Fink, 2001) for 30 min under shaking conditions (900 r.p.m.). A sample of 100 μl was transferred to a new well to determine the OD_{570} in a microtitre plate reader. If necessary, dilutions were made in 10% SDS. Total biomass was determined using the *DC* protein assay (Bio-Rad) after treating the material at 100°C for 30 min in 0.2% SDS–1 M NaOH. Bovine serum albumin (BSA) was used as a standard.

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